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Supporting Information

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for

Introduction of an Aliphatic Ketone into Recombinant Proteins in a Bacterial Strain that Overexpresses an Editing-Impaired Leucyl-tRNA Synthetase

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Experimental Methods

Materials: Amino acids **1** and **4** were obtained from Sigma (St. Louis, MO). Amino acids **2** and **3** were prepared by alkylation of diethyl acetamidomalonate with the alkyl tosylate or alkyl bromide, followed by hydrolysis, decarboxylation and deacylation.[1] Amino acid analogs **2** and **3** were used as the racemates in both in vitro and in vivo assays. [^{32}P]-radiolabeled sodium pyrophosphate was purchased from NEN Life Science Products, Inc (Waltham, MA). Oligonucleotides were synthesized at the Caltech Biopolymer Synthesis Center (Pasadena, CA). General cloning was performed in XL-1 blue cells (Stratagene). Expression strain SG13009 was obtained from Qiagen.

Synthetase expression and purification: *E. coli* LeuRS was purified from SG13009 cells transformed with the plasmid p32leus[2] (which encodes the synthetase under the control of an IPTG-inducible promoter), and with the pREP4 repressor plasmid. Protein expression was induced at OD_{600} = 0.6 by addition of IPTG to a final concentration of 0.5 mM. Cells were harvested after 3 hours by centrifugation and lysed by sonication. The enzyme was isolated using Ni-NTA agarose resin under native conditions according to the manufacturer's instructions (Qiagen). Proteins were stored in Buffer A/glycerol (50 mM Tris-HCl, 1 mM DTT, 50% glycerol). Aliquots were flash frozen in liquid nitrogen and stored at -80℃. The concentration of each enzyme was determined by measuring the absorbance at 280 nm under denaturing conditions.

ATP-PP$_i$ exchange assay: Amino acid activation was monitored by an amino acid dependent ATP-pyrophosphate (PP$_i$) exchange assay at room temperature[3] in buf-

fer containing HEPES (50 mM, pH 7.6), $MgCl_2$ (20 mM), DTT (1 mM), ATP (2 mM), [^{32}P]-PP_i (0.5 TBq mol^{-1}, 2 mM), and LeuRS (75 nM). The amino acid concentrations varied depending on the activity of the enzyme toward the substrate. Reactions were initiated with the addition of LeuRS. Aliquots (20 µL) were quenched using a standard quench solution (500 µL) containing PP_i (200 mM), $HClO_4$ (7% w/v), and activated charcoal (3% w/v). The charcoal was washed twice using a solution of PP_i (10 mM) and $HClO_4$ (0.5% w/v), and counted using a scintillation counter. Kinetic parameters (k_{cat} and K_m) were determined by nonlinear regression analysis of curve-fitting of the Michaelis-Menten equation. The results reported in Table 1 are averages from triplicate experiments.

Construction of expression plasmids: The expression plasmid pA1EL was constructed as described by Tang et al.[2] It encodes a synthetic leucine zipper protein A1 under control of an inducible T5 bacteriophage promoter and the *E. coli leuS* gene with its endogenous promoter. The mutations at position T252 of *leuS* were introduced by overlap-extension-PCR mutagenesis using the Quikchange site-directed mutagenesis kit (Stratagene) and pA1EL as a template. The primer pairs were used to generate the tyrosine, isoleucine and valine mutations. The integrity of the coding regions was confirmed through DNA sequencing. The plasmids carrying the T252Y, T252I, and T252V mutants were designated as pA1T252Y, pA1T252I, and pA1T252V respectively. Overexpression of LeuRS was verified by SDS-PAGE of whole cell lysates from overnight cultures.

Analog Incorporation Assay: The leucine auxotrophic strain LAM1000[4] was transformed with pA1EL, with pA1T252Y (or pA1T252I or pA1T252V), and with pREP4 to yield the A1 expression strains LAM1000/(pA1EL, or pA1T252Y, or pA1T252I, or pA1T252V)/pREP4. M9 (200 mL) containing 20 canonical amino acids (40 mg L^{-1}), $MgSO_4$ (1 mM), $CaCl_2$ (1 mM), glucose (0.4% *w/v*), thiamine (5 mg mL^{-1}), ampicillin (200 mg mL^{-1}), and kanamycin (25 mg mL^{-1}), was inoculated with an overnight culture (1 mL) of the expression strain. When the turbidity of the culture reached an OD_{600} between 0.9 and 1.0, the cells were sedimented and the cell pellet was washed with salt solution (0.9% NaCl in H_2O) three times. The cells were resuspended in fresh M9 medium with 16 natural amino acids (M9 –LIVM medium). Leu, Ile, Val, and Met were not added to the medium (Ile, Val, and Met were omitted in order to reduce background expression levels as previously described).[2] Test tubes containing aliquots (10 mL) of the resulting culture were prepared and supplemented with **2** (100

mg L^{-1}), **3** (200 mg L^{-1}) or **4** (320 mg L^{-1}). Leucine (40 mg L^{-1}) was added to a separate culture (10 mL) as a positive control. A culture without additional amino acids (or any analog) served as the negative control. Protein expression was induced by addition of IPTG to the culture at a final concentration of 1 mM. Cultures were grown for 3 h and the cells were collected by centrifugation (5000g, 10 min, 4℃), resuspended in Buffer B (600 µL, 8.0 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.0) and frozen at -80℃. Protein expression was monitored by SDS-PAGE and visualized by Coomassie Brilliant Blue staining.

Protein composition analysis: The target protein A1 was purified from cells resuspended in Buffer B (600 µL) by using Ni-NTA spin columns (Qiagen) according to the manufacturer's instructions. A1 protein was eluted in Buffer B (400 µL), pH 4.5. A portion of the eluent (10 µL) was diluted in ammonium bicarbonate (90 µL, 50 mM $(NH_4)_2CO_3$). Trypsin stock solution (5 µL, 0.1 µg μL^{-1}) was added, and the sample was incubated at room temperature overnight. The reaction was quenched by addition of trifluoroacetic acid (TFA, 2 µL). The reaction mixture was subjected to C18 ZipTip (Millipore) purification, and peptide fragments were eluted (3 µL) in a solution of TFA (0.1%) and CH_3CN (50%). A portion (1 µL) of C18 ZipTip eluent was used for tryptic MALDI analysis. The remaining volume of spin column eluent (390 µL) was dialyzed against water extensively and lyophilized to a fluffy powder. The powder was sent directly for MALDI mass spectrometry and amino acid analysis.

Modification of Ketone Side Chains: Purified proteins containing ketone functionalities were dissolved in PBS buffer (200 µL, pH 6.0) and added to biotin hydrazide (BH, 20 µL, 5 mM) in PBS. Protein/BH mixtures were incubated at room temperature for 1 to 1.5 h. Reaction solutions were then washed twice with distilled water using a buffer-exchange column (Millipore, MWCO = 5 kDa). Standard western blotting procedures were used to identify proteins modified with BH.

References

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